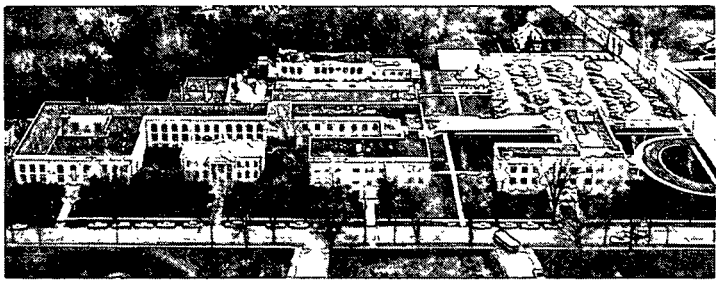

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

IPC TECHNICAL PAPER SERIES
NUMBER 115

CONIFER SUSPENSION CULTURE MEDIUM DEVELOPMENT USING ANALYTICAL DATA FROM DEVELOPING SEEDS

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NOVEMBER, 1981

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by

J. D. Litvay, M. A. Johnson, D. Verma, D. Einspahr, and K. Weyrauch[1]

ABSTRACT

This investigation emphasizes the chemical environment of the various zones of the immature conifer seed prior to fertilization for the purpose of defining the nutritional requirements of conifer cells in vitro. An elemental analysis of these zones (the archegonium, the erosion zone, and the gametophyte) revealed that none of the plant tissue culture media formulations published to date were adequately balanced to grow and maintain conifer cells in culture.

Our initial attempt to correct this imbalance has resulted in a culture medium formulation which has allowed tissues from juvenile seedlings or mature trees of Douglas-fir (Pseudotsuga menziesii) and loblolly pine (Pinus taeda) to be grown and maintained as fine cell suspensions for prolonged periods. Preliminary work in our laboratory suggests that the concepts leading to the formulation will find widespread use in work on coniferous as well as other difficult-to-culture species. The media formulation and other pertinent data related to a hypothesis on natural embryogenesis are included.

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[1]The authors are members of The Institute of Paper Chemistry's conifer tissue culture research team. Additional members of this team include S. Verhagen, J. Carlson, R. Feirer, G. Mignon, and H. Kaustinen. The authors wish to acknowledge that Dr. Donald Durzan was project leader when some of the reported research was being conducted.

INTRODUCTION

Investigations into natural embryogenesis of Douglas-fir and loblolly pine revealed that the natural embryo encounters several successive changes in its chemical environment during development (Technical Paper Series No. 114). Development of an appropriate series of medium changes for in vitro somatic embryogenesis is under investigation, based upon data obtained from analysis of developing seeds of the above species. The following information describes the data which have allowed us to better understand the sequence of events occurring in natural embryogenesis and which have enabled us to formulate a new gymnosperm cell suspension medium.*

MODEL SYSTEMS RESULTS

The biochemistry and histology of natural embryogenesis are extremely complicated. To better understand the sequence of events in natural embryogenesis described earlier in IPC Technical Paper Series No. 114, data like those given in Table I should be collected. Some of the actual data collected are given below.

Embryo and Seed Statistics

Table II lists some of the pertinent weight data on natural and wild carrot somatic embryo development. This information is important because it will be required in subsequent media formulations.

Inorganic Analysis of Natural Douglas-fir Seeds During Embryogenesis

Inorganic elemental analysis of the three zones present in an unfertilized immature Douglas-fir seed is shown in Table III. Also shown are inorganic analyses of mature embryos, mature gametophytic tissues, and seedlings.

*Reported results are part of the Institute's Project 3223, The Mass Production of Conifer Hybrids.

TABLE I

MODEL PARAMETERS[a]

Parameter	Archegonium	Erosion Zone	Modified Erosion Zone
System			
Open/closed			
Temperature			
Light			
Humidity			
Movement			
Embryo development			
Cell size			
Cell number			
Growth rate			
Doubling number			
Cell ultrastructure			
Meristematic/other			
Nucleus configuration			
Mitochondria			
Ribosomes			
Plastids			
Microbodies			
Membranes			
etc.			
Media			
pH			
Osmolarity			
Redox potential			
Solids content			
Macronutrients			
Micronutrients			
Vitamins			
Growth regulators			
[Amino acids]			
[Carbohydrates]			
[Lipids]			
[RNA/DNA]			
etc.			

[a]This figure is representative of a concept and is not intended to represent results.

TABLE II

SEED AND EMBRYO WEIGHT DATA

I. Comparison of seed weights

Species	Seeds/lb
Douglas-fir	30,000
Loblolly pine	20,000
Carrot	368,000

II. Comparison of embryo weights

Natural Embryo

Douglas-fir	0.6 - 1.0 mg/embryo (o.d.)
Loblolly pine	0.9 - 1.1 mg/embryo (o.d.)

Somatic Embryo

Wild carrot	0.01 mg/embryo (o.d.)

0.5 µL inoculum yields approximately 600 embryos weighing a total of approximately 6 mg (o.d.)

III. Comparison of Douglas-fir and Loblolly Pine Natural Systems

Weight of Douglas-fir and Loblolly Pine Seeds Prior to Fertilization

	Douglas-fir mg (o.d.)	Loblolly Pine mg (o.d.)
Archegonium	0.08	0.07
Erosion zone	0.04	0.08
Gametophytic tissue	0.38	0.35

Weight of Douglas-fir and Loblolly Pine Mature Seed

	Douglas-fir mg (o.d.)	Loblolly Pine mg (o.d.)
Gametophytic tissue	7-8	8-9
Embryo	1	1

TABLE III

INORGANIC ANALYSIS OF DEVELOPING DOUGLAS-FIR SEEDS (μg/g o.d.)

Element	Prefertilization			Mature Embryo	Mature (G)	Seedling Values	Embryo Uptake	Available in A and E Zones	Total Present in Immature Seed
	Archegonium (A)	Erosion Zone (E)	Gametophyte (G)						
K	40,000	55,000	57,000	4,000	10,000	7,200	0.4-4.0	4.4	26.0
Ca	500	690	200	250	150	6,800	0.005-0.05	0.0055	0.0836
Cl	--	--	--	--	--	--	--	--	--
Na	6,000	6,200	2,900	100	400	1,100	0.01-0.1	0.60	1.70
Mg	3,000	2,800	2,200	2,400	2,800	3,000	0.02-2.4	0.30	1.13
Mn	60	50	45	100	250	52	0.01-0.1	0.0057	0.0198
Fe	1,150	2,000	620	100	75	690	0.01-0.1	0.140	0.163
Cu	<50	130	<50	<50	<50	12	0.005-0.05	0.0074	0.0264
Zn	400	1,500	<50	<50	200	86	0.005-0.05	0.0428	0.062
B	100	760	100	50	<50	52	0.005-0.05	0.0298	0.0678
Mo	--	--	--	--	--	--			
Co	--	--	--	--	--	--			
P	7,500	8,600	7,700	5,700	2,400	1,400	0.57-5.7	0.783	3.51

From these data one can obtain the following important information: (1) the radically different nutritive compositions of the different zones encountered by the embryo during development (Table III, Fig. 1); (2) nutrient uptake levels in the mature embryo; (3) the amount of nutrition available to the embryo from the hydrolyzed archegonium and erosion zone tissues; (4) estimates of the kinds and amounts of elements present in the immature seed *versus* the mature seed and, therefore, an estimate of the composition of the influxing media; and (5) nutritional information on gymnosperm embryos that can be used for media reconstitution and media comparison (actual media formulations to come later).

Moisture Content and Weight Gain in Developing Loblolly Pine Seeds During Embryogenesis

Loblolly pine and Douglas-fir natural embryos develop along similar paths with respect to growth curves as well as changes in moisture contents and seed weights during development. Table IV gives the moisture contents for the three zones found in a loblolly pine seed immediately prior to fertilization. Table V gives the weight gain and moisture content changes for loblolly seeds as the embryo develops within the seed. Figure 2 is a plot of embryo growth, moisture content change, and weight gain for loblolly pine.

The pH of Natural Douglas-fir and Loblolly Pine Seeds During Embryogenesis

Besides the analysis conducted above, pH was monitored during natural embryo development. The pH is an important component in many chemical reactions and all media formulations. Also, pH can control such factors as availability of substrates and rates of reactions where enzymes are concerned.

In vivo pH is not an easy parameter to measure, but we were interested only in large changes in the various environments that the embryo encounters and not in measuring intracellular pH. Thus zonal pH changes were determined by pressing pH

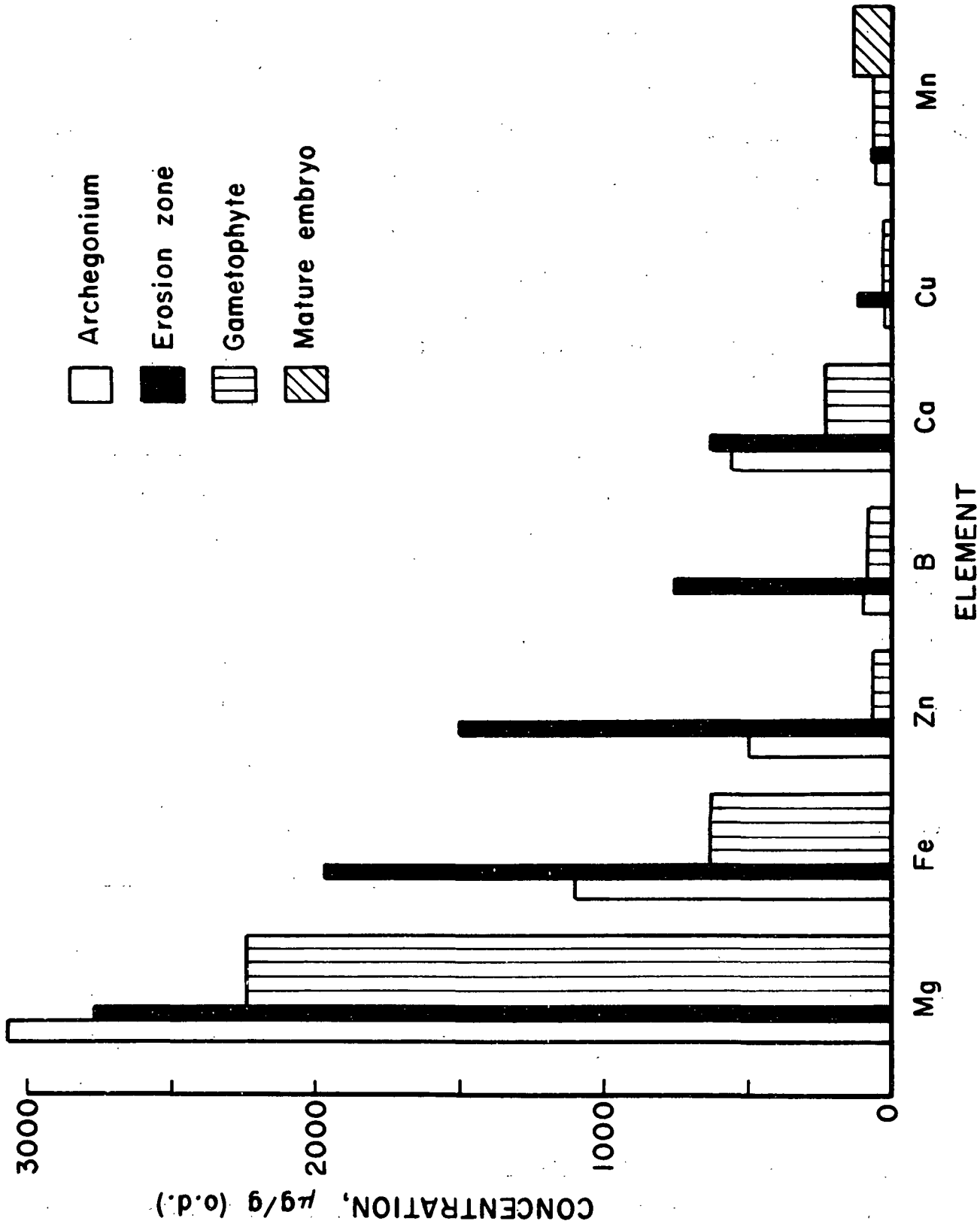

Figure 1. Zonal elemental concentrations present in immature Douglas-fir seeds.

paper against freshly cut surfaces that exposed the tissue zones requiring measurement. We understand the limitations of such a system but feel it has generated useful results. Figure 3 shows the pH values of the different zones with time as the embryo develops. Figure 4 is a plot of pH versus embryo development showing the pH of the environments that the embryo encounters as it develops.

TABLE IV

MOISTURE CONTENT OF IMMATURE SEED ZONES IMMEDIATELY PRIOR TO FERTILIZATION

	Average			
Zone	Fresh Wt. mg/seed	Dry Wt. mg/seed	M.C., %	Solids Content, %
Archegonium	0.125	0.070	44	56
Erosion zone	0.236	0.085	58	42
Gametophytic tissue	2.95	0.350	89	11
Seed coat	34.70	10.55	70	30

TABLE V

WEIGHT AND MOISTURE CONTENT CHANGES OF DEVELOPING LOBLOLLY PINE SEEDS

	Weeks after Fertilization					
Parameters	0	2	4	6	8	10
O.d. wt., mg/seed	0.505	0.625	0.890	2.92	5.18	7.72
Wt. water, mg/seed	2.80	5.61	5.19	4.58	5.22	4.74
Green wt., mg/seed	3.30	6.23	6.05	7.50	10.40	12.46
M.C., %	85	90	85	65	50	38

Interpretation of Events in Natural Embryogenesis

Our hypothesis is that the environment (medium) of the archegonium is optimum for the production of small coherent masses of cells, herein referred to as

proembryonic masses (PEM's), and can be considered to function as such. Therefore, a medium based upon the archegonial environment should allow one to obtain and maintain a fine suspension of cells similar to PEM's.

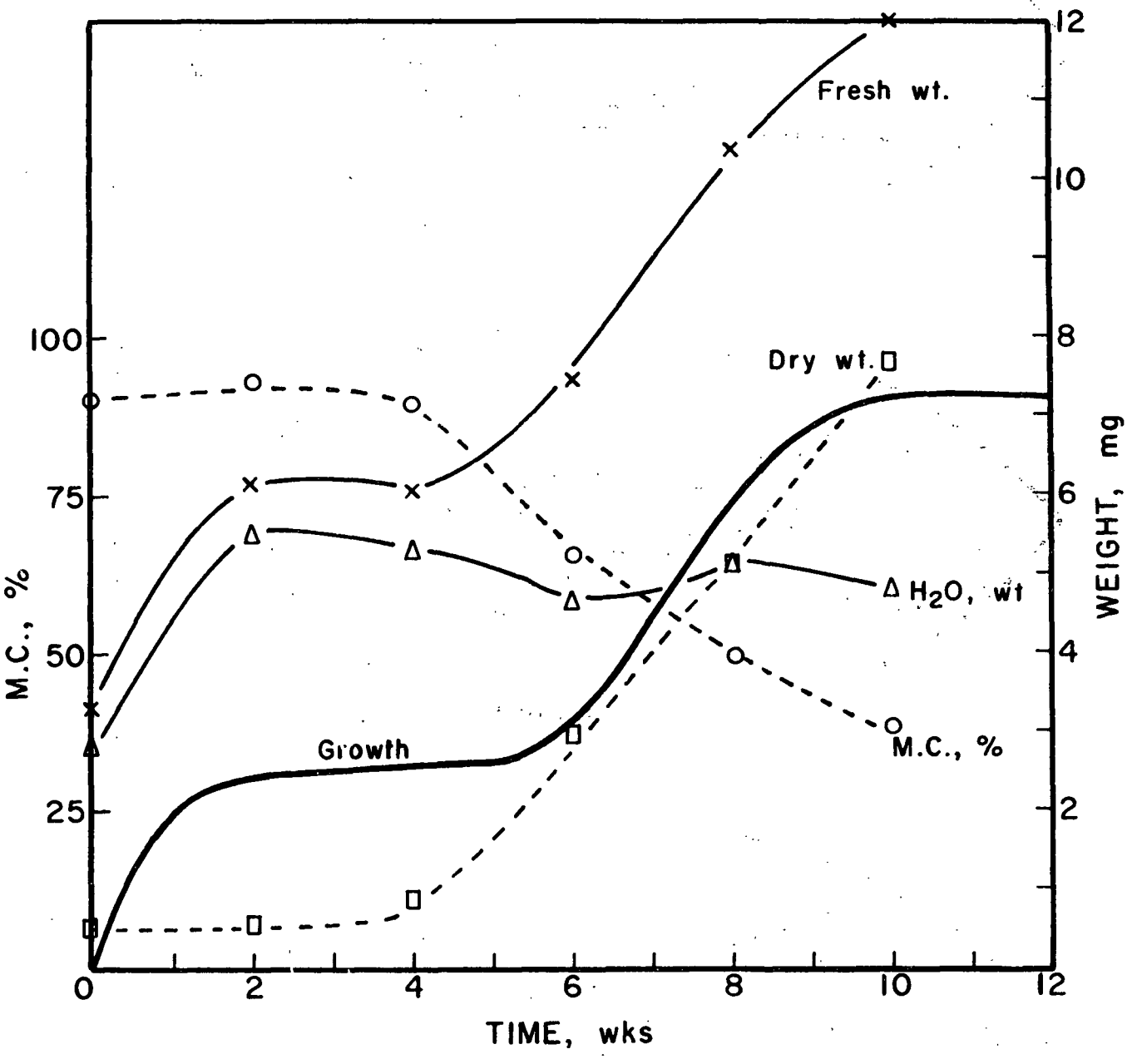

Figure 2. Weight and moisture changes in developing loblolly pine seeds.

Further, it is our contention that the erosion zone is inhibitory to growth, probably due to its high concentration of microelements and its lower pH.

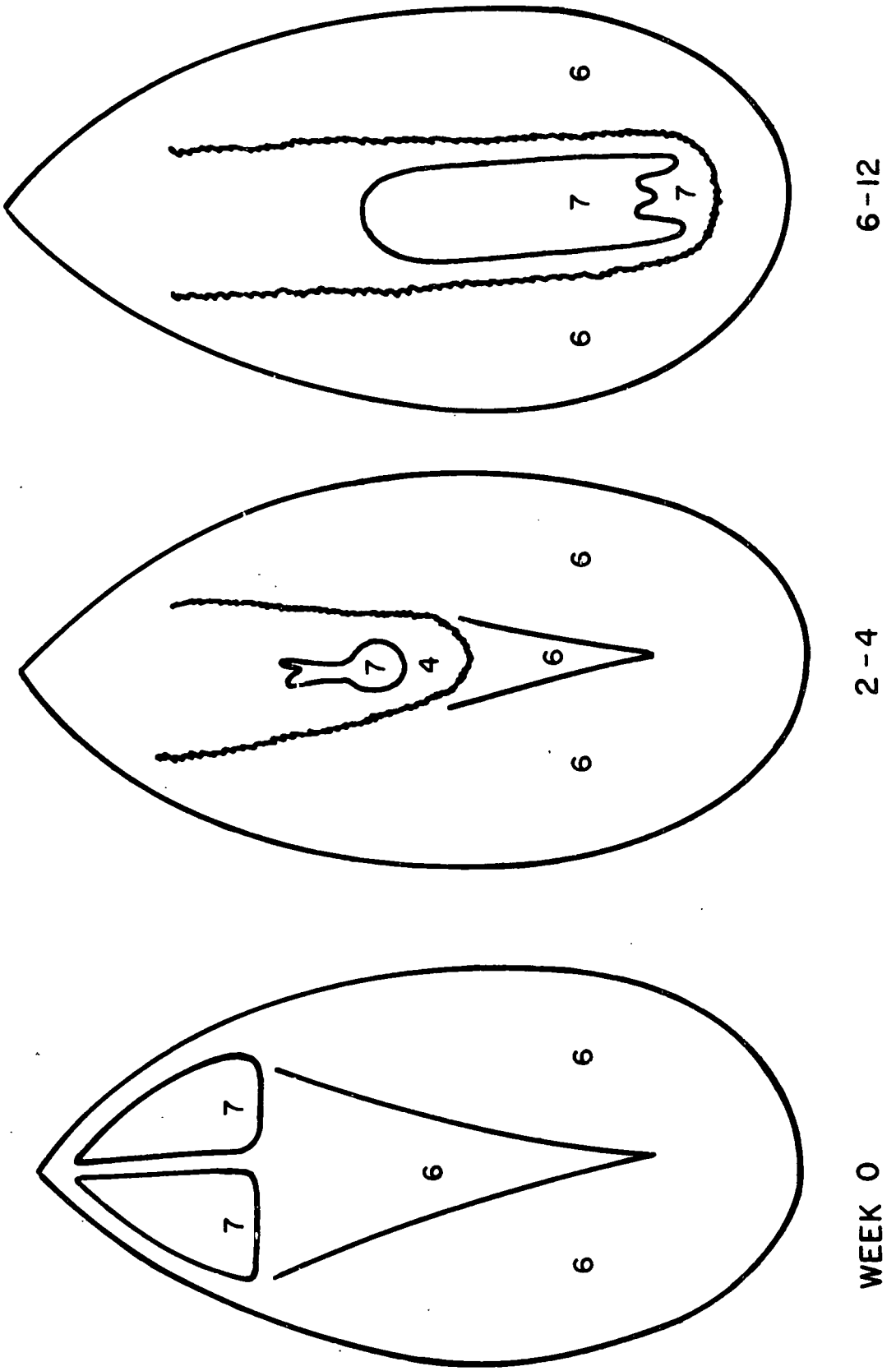

Figure 3. Zonal pH changes in developing loblolly pine seeds.

The probable function of this zone is to allow the preformed clump to build up its intracellular resources for the impending growth cycle which results in internal cell divisions and in compartmentalization of the structure. Compared with the archegonium, the erosion zone contains significantly higher concentrations of starch, Cu, B, Fe, and Zn; the first four factors tend to influence the energy system of the cell.

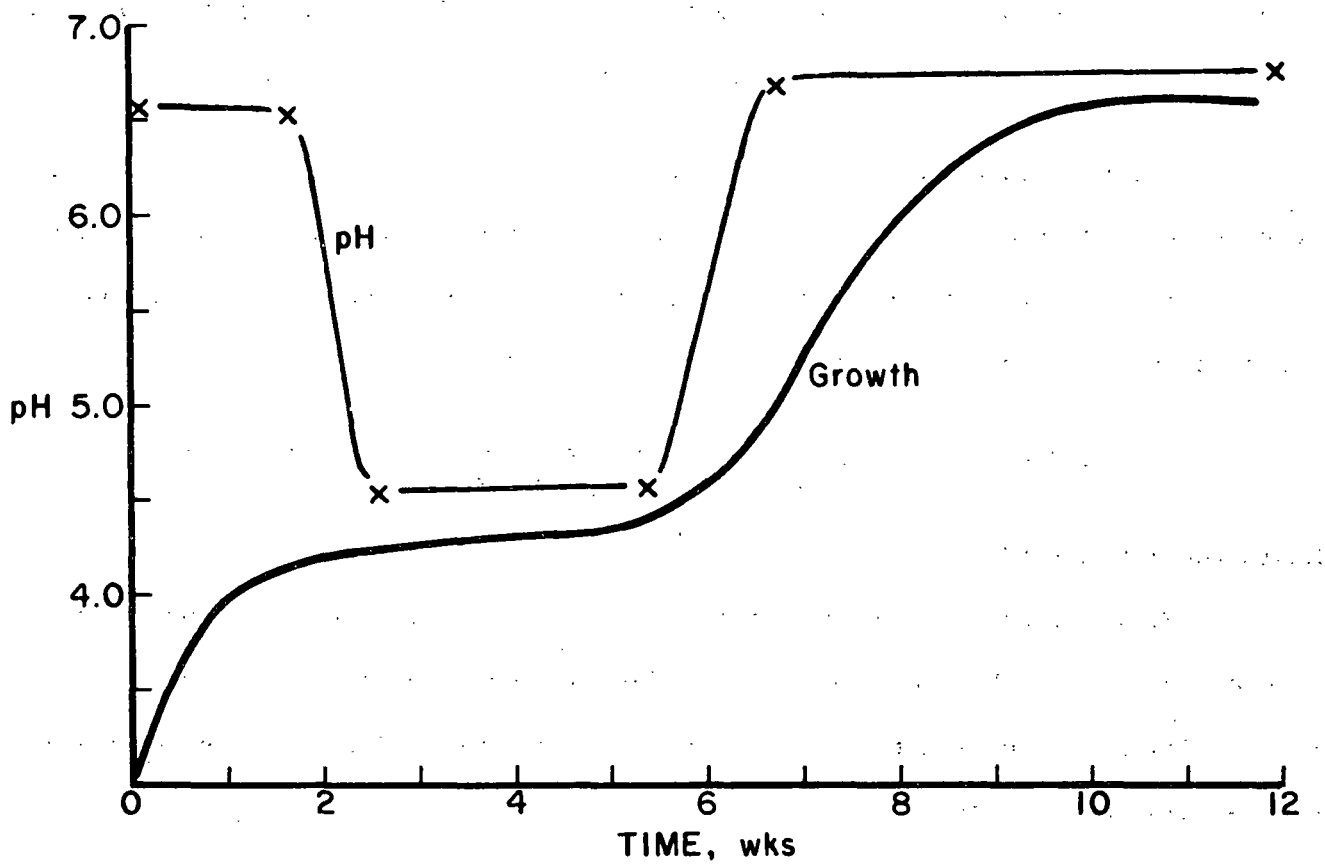

Figure 4. Measured pH fluctuations encountered by loblolly pine embryos during development.

Observation of natural embryogenesis at the launch stage (polarized growth) demonstrates that the environment of the embryo is altered by (1) dilution of the high solids content and high micronutrient medium of the erosion zone by the lower solids content and lower micronutrient medium of the gametophytic zone, and (2) an influx of nutrients from outside of the seed. An analysis of the preexisting

environment and of the influxing nutrients reveals the following: (1) dilution brings the concentrations of those elements that were probably inhibitorily high in the erosion zone (Cu, Fe, B) down to optimal levels and those elements at or near optimal levels (K, Ca) to suboptimal levels; and (2) the influxing nutrients increase the pH to neutral and bring with them large amounts of Mn and N, thus changing the C/N, Mg/Mn, and Zn/Mn ratios. The influxing nutrients also bring in with them a large amount of organic material such as DNA and RNA degradation products, which may have specific regulatory roles similar to the cytokinins.

The speculated net effect of these changes would be to increase the pH, increase energy production, alter the growth regulator status from a high auxin to a lower auxin level (possibly a high cytokinin level), and promote polarization due to stresses caused by dilution or osmolarity reduction.

USE OF MODEL SYSTEM INFORMATION TO OBTAIN AND MAINTAIN CONIFER CELL SUSPENSIONS

MEDIA CONSIDERATIONS

Having presented an interpretation of the data obtained from our studies on natural (zygotic) embryogenesis in a conifer, we must now apply these concepts to the nutritional requirements for growing and maintaining fine cell suspensions of conifer tissues in vitro.

To our knowledge, there have been no other studies attempting to determine the nutritional requirements for growing conifer cells based on the composition of dissected immature seeds. As a consequence, until now the plant tissue culture media specifically developed for growing herbaceous angiosperms have been used for growing coniferous species as well. For discussion and comparison purposes we will use the medium developed by Murashige and Skoog (M.S. medium), the elemental composition of which appears in Table VI. We have also included in Table VI the

TABLE VI

COMPARISON OF WILD CARROT AND MURASHIGE-SKOOG MEDIA ELEMENT CONCENTRATIONS TO THE NEEDS OF GYMNOSPERM EMBRYOS

Element	W.C. Medium, µg/mL	M.S. Medium, µg/mL	Archegonium Zone, µg/mL	Erosion Zone, µg/mL	Gametophyte, µg/mL	Mature Embryo, µg/mL	Embryo Uptake, µg/embryo	Max. I.D. per 1 mL M.S.
K	1574	782	400	555	576	40.4	0.4-4.0	200
Ca	60	121	5.0	6.97	3.03	0.51	0.005-0.5	2000
Na	2	4.6	60	62.6	29.3	1.0	0.01-0.1	<1
Cl	462	212						
Mg	18	36.8	30	28.3	22.2	24.2	0.2-2.4	15
Mn	2	5.51	0.6	0.5	0.45	1.0	0.01-0.1	53
Fe	4	5.57	11.5	20	6.2	1.0	0.01-0.1	70
Zn	1.36	1.92	4	15	<0.5	<0.5	0.005-0.05	72
Cu	0.004	0.006	<0.5	1.3	<0.5	<0.5	0.005-0.05	<1
B	0.0425	1.08	1.0	7.6	1.0	<0.5	0.005-0.05	22
Mo	0.0001	0.096						
Co	0	0.006						
N	482	840						
P	29.6	39	75	86	77	58	0.57-5.7	12
C	30,000	30,000						
Solids content	2.5		56	42	11			

composition of the wild carrot (W.C.) medium used by Wetherell, because we use the wild carrot system as a model when studying somatic embryogenesis.

Now let us compare these angiosperm media with the reconstituted medium representative of the various chemical environments (zones) of the immature Douglas-fir seeds (Table VI). Based on our hypothesis, the zone to be considered as the most relevant would be the "archegonium," which supports the active division of proembryonic single cells after fertilization. Comparison of the reconstituted archegonium zone with M.S. or W.C. media show significant differences in many element concentrations, thus indicating that standard W.C. and M.S. media would not be optimal for growing gymnosperm cells as suspensions.*

A second important determination, once we have taken care of cell maintenance needs, is the nutritive requirements for growing these cells into embryos. Whether or not standard media formulations are concentrated enough to grow gymnosperm embryos can also be answered in Table VI. Table VI lists the amounts of each element required to grow one Douglas-fir embryo; also listed are the amounts of each element present in one milliliter of M.S. medium. From these numbers one can calculate (assuming 100% uptake) the maximum number of natural Douglas-fir embryos that could be grown in 1 mL of M.S. medium, assuming the weight of one embryo to be 1 mg. It should be noted that certain elements such as Cu are found in extremely low concentrations in the M.S. and W.C. media. A deficiency situation will arise in these media if (1) uptake efficiency is less than 100% (which it usually is), (2) medium strength is reduced, or (3) inoculum density (I.D., number of clumps placed in the medium) is greater than the medium can support (i.e., the level of Cu in the M.S. medium is such that it will allow only 1 clump per mL to grow into an embryo, whereas normally 100-1000 clumps are placed in 1 mL of medium).

*This seems to be the case, since obtaining and maintaining gymnosperm cells as fine suspensions for prolonged periods has not been reported by any other laboratory.

Thus, if we are using a closed batch system like that of the wild carrot, it is highly unlikely that any PEM's of Douglas-fir could be grown successfully into embryos. Obvious ways around such a problem are (1) use of an open system or (2) frequent media changes to replenish spent medium. The essential point to be made is that the strategy employed to obtain embryogenesis must balance cell and embryo needs against medium formulation and inoculation density (I.D.).

Our initial attempts to tackle the above considerations have led to the formulation of a new medium, Litvay's medium (LM), whose formulation is based on the analysis of the archegonium of the immature Douglas-fir seed. This formulation, as shown in Table VII when compared with the M.S. and W.C. media, attempts to (1) reorder the individual elements into the proper ratios for gymnosperm cells and then (2) adjust the concentrations of the elements to allow these cells to grow into embryos (assuming I.D. and screen size fractions are adjusted to balance this gymnosperm system; this concept will be explained more fully in another paper in this series).

With this medium (LM) we have been able to obtain and, more importantly, maintain Douglas-fir and loblolly pine cells in liquid suspension and as calli from both juvenile and mature sources (see Table VIII). This initial medium formulation is being tested with specific alterations to see if any further improvements can be made.

In addition, preliminary studies in our laboratory indicate that LM, along with its modifications deciphered in Table VII, will find widespread use in tissue culture studies for the following reasons:

1. LM will elevate the growth and subculturability potential of conifer tissue or tissue cultures growing suboptimally on other media such as the M.S. medium.

TABLE VII

LITVAY'S MEDIUM (LM) FORMULATION[a]

Compound	Levels, mg/L	Compound	Levels, mg/L
NH_4NO_3	1650.0	$CuSO_4 \cdot 5H_2O$	0.50
KNO_3	1900.0	$CoCl_2 \cdot 6H_2O$	0.13
$MgSO_4 \cdot 7H_2O$	1850.0	$FeSO_4 \cdot 7H_2O$	27.8
KH_2PO_4	340.0	Na_2EDTA	37.3
$CaCl_2 \cdot 2H_2O$	22.0	Myoinositol	100.0
KI	4.15	Nicotinic acid	0.5
H_3BO_3	31.0	Pyridoxine · HCl	0.1
$MnSO_4 \cdot H_2O$	21.0	Thiamine · HCl	0.1
$ZnSO_4 \cdot 7H_2O$	43.0	Sucrose	30,000.0
$Na_2MoO_4 \cdot 2H_2O$	1.25		

[a]Media comments - many different alterations of this medium formulation have been tested on Douglas-fir and loblolly pine. The following are some of the results, with the ranges or modifications that have been tested. (1) Copper levels 10 to 15-fold higher cause no apparent damage; 50-fold increases are toxic. (2) Magnesium levels may be somewhat lowered (1/2), and part of the magnesium can be supplied as $Mg(NO_3)_2$. The latter will result in the reduction of $SO_4^=$ ions. (3) The calcium levels can be increased to near M.S. levels, but cell quality (as determined by cell origin from the explant) and subculturability may be affected. (4) Manganese levels may be decreased. (5) Vitamin levels can be increased. (6) Sucrose levels as high as 6% (w/v) have been successfully used to support higher growth rates. (7) Alteration of the amount and forms of nitrogen have been successfully tested (reduction of ammonium nitrate, supplementation with amino acids, etc.). (8) Suspensions have been successfully initiated and maintained using different growth regulators and different concentrations of growth regulators. Among auxins 2,4-D or NOAA (β-naphthoxyacetic acid) have been used at various levels with and without a cytokinin. Use of NAA, with or without a cytokinin, was not as satisfactory. The best results were obtained with approximately 2.5 ppm of 2,4-D, with or without a cytokinin, and 5.0 NOAA, with a cytokinin. (9) Alteration of initial pH or NH_4^+ content may be necessary for pH control; pH should not be allowed to drop below 4.0 during liquid suspension subculture. (10) Zn and Fe modifications are currently being tested.

2. LM is specifically suited for generating fine gymnosperm suspension cultures.

Therefore, it is expected that this medium will have applications in

a) studies aimed at producing biochemicals from suspension cultures,

b) developmental studies on somatic embryogenesis or studies on plant morphogenesis in suspension cultures or agar,

c) studies on mature tissues from the gymnosperms.

3. Current literature shows that tissue culture studies on cereal species are hampered by the lack of a suitable culture medium. From our preliminary studies, we find that even the tissues from cereal species such as Avena sativa and Zea mays will grow better on LM than on M.S.

TABLE VIII

STATUS OF CELL LINES GROWING IN LM

	Explant Source[a]					Auxin			Calcium	
	C[a]	H	S	N	M	NOAA	NAA	2,4-D	Hi	Lo
Douglas-fir										
juvenile	/[b]	/				/	/	/	/	/
mature			/	X[c]		X	/	X	/	X
Loblolly pine										
juvenile	X		X	X		X		X	X	X
mature				/	X	/	/	X	/	X

[a]C-cotyledon, H-hypocotyl, S-stem, N-needle, and M-megasporophyll.
[b](/) cell suspensions obtained and maintained for less than 6 months but still under study.
[c](X) cells maintained for at least 6 months as liquid cell suspensions by sub-culturing every two weeks.